

AXIMA-TOF²[™]

Simultaneous Protein Identification and
Relative Quantification of Complex
Mixtures using iTRAQ[™] methodology,
LC-MALDI and AXIMA-TOF²[™]

- Proteins from a complex mixture unambiguously identified and quantified using iTRAQ[™] reagents
- High energy CID MS/MS provides well balanced information rich fragment ion spectra
- Low Mass Zoom function enhances iTRAQ[™] reporter ion intensity for improved statistics during quantitation

Simultaneous Protein Identification and Relative Quantification of Complex Mixtures using iTRAQ™ methodology, LC-MALDI and AXIMA-TOF²™

Introduction

An important component of proteomic research is the identification of perturbation of normal cellular protein levels, which is vital for understanding disease processes and intervening to control disease. In order to achieve these goals it is necessary to separate, quantify, identify and characterize individual proteins of interest. It is also highly advantageous if all these requirements can be fulfilled in a single experiment. The iTRAQ™¹ stable isotope tagging methodology (isobaric tags for relative and absolute quantification) combined with mass spectrometric analysis with the AXIMA-TOF²™ LC-MALDI functionality allows this. The analysis allows the simultaneous measurement of expression profiles of multiple samples, such as normal versus diseased versus drug-treated states in a single investigation. A diagram of the experimental technique is shown in Figure 1.

iTRAQ™ relative quantification chemistry, which targets primary amine groups, produces isobaric parent ions, therefore, the peptide precursor masses and the MS/MS fragment ion masses are the same for differentially labelled samples. These data can easily be submitted for database searching and provide protein identification. Simultaneously, sample dependent reporter masses, used for quantification purposes, are apparent in the MS/MS spectrum in the low mass region at 114 - 117 Da.

Complex protein mixtures can be identified and quantified using an iTRAQ™ 'shotgun' proteomics approach. LC-MALDI is a technique that can easily and effectively deal with such complex biological samples. The experimental technique combines an LC separation of digested proteins with online fractionation directly onto a MALDI target. Fractionation and matrix addition is performed automatically by a spotting robot such as the Accuspot™. The spotting probe simultaneously delivers matrix solution and the LC eluent, optimizing the mixing of the two solutions and minimizing matrix 'hot spots'. MALDI mass spectrometry analysis is then performed offline. The analysis can be performed under full automation on a novel MALDI TOF-TOF mass spectrometer, the AXIMA-TOF²™, using purpose designed, dedicated LC-MALDI software.

Here we discuss the combination of AXIMA-TOF²™ and iTRAQ™ methodology for the systematic comparison of differentially labelled samples to facilitate the investigation of protein function or disease states.

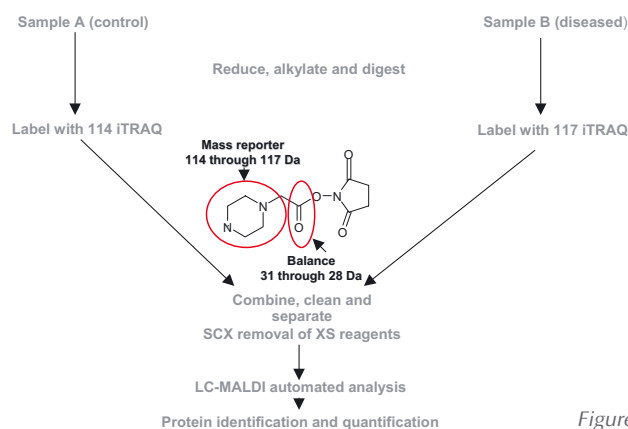


Figure 1. iTRAQ™ workflow

Methods

Materials: A mixture of five tryptically digested proteins was prepared. Two equimolar aliquots of the sample were subjected to iTRAQ™ labelling with reagents 114 and 117 respectively according to the manufacturer's instructions. A pooled aliquot of the modified peptides was cleaned up with an SCX trap followed by a 1D C₁₈-RPLC run.

LC and Accuspot™ conditions: A 5 µl aliquot of the sample diluted in mobile phase A (500 fmol per protein) was loaded directly onto the column (300 µm i.d. x 15 cm, 3 µm, Pepmap C₁₈ (LC Packings)) and separated using a two stage linear gradient: (A = 5% acetonitrile + 0.05% TFA, B = 80/20 acetonitrile + 0.05% TFA)

The flow rate was set at 0.2 ml/min pre-split, giving a flow rate of ~5 µl/min post-split. The eluent passed through a UV/Vis detector (220 nm) and was mixed with α-cyano 4-hydroxycinnamic acid matrix (CHCA; 5 mg/ml in 50/50 acetonitrile/0.1% TFA) and deposited onto a stainless steel MALDI target using the Accuspot™ LC-MALDI deposition robot. Samples were deposited across a 384-well MALDI target at a rate of 6 sec/spot producing ~1 µl spots.

MALDI TOF-TOF: Peptide mass fingerprints and peptide ion MS/MS spectra were acquired automatically on an AXIMA-TOF²™ MALDI mass spectrometer (Shimadzu Biotech, Manchester, UK) in positive ion mode. The dedicated LC-MALDI automation software was configured to allow peak picking of any precursor above a minimum intensity threshold of 30 mV from chromatographic peaks of width of at least 20 seconds. All precursors that met acceptance criteria were selected for MS/MS (i.e. no restriction of the number of MS/MS acquisitions was imposed). Peptide ions were isolated from samples using an innovative ion gate, the monoPULSE™, allowing the selection of precursor ions with an accuracy of ±1 Da, a resolution of approximately 400 FWHM (at 1000 Da) eradicating the issue of precursor ions in close proximity. High energy fragmentation was performed by means of a CID cell positioned in the ion beam. Helium was used as the collision gas.

Mascot® database searching:

- Searched against the NCBI nr database using Mascot® (www.matrixscience.com)
- Taxonomy 'all'
- Enzyme was specified as 'trypsin' (allowing for 1 missed cleavage)
- 42 fragment ions were selected from each MS/MS spectrum over six segments (2-98% of precursor mass)
- A search tolerance of ± 0.5 Da/ ± 0.8 Da (parent/fragments) was used to search the MS/MS data

Results

An aliquot of the labelled samples was analyzed in MS mode on the AXIMA-TOF2™ - the resulting peptide mass fingerprint is shown in Figure 2. The peptide mass fingerprint was highly complex and when the peptide masses were searched against the NCBI nr database no protein hits were returned.

The derivatized mixture was then subjected to a simple 1D RP HPLC separation, followed by automatic deposition with matrix over a single 384 well MALDI target plate. Following the analysis, the LC-MALDI software produces a visualization of the MALDI target plate, highlighting by a sliding colour scale, intensity of the peptide ions contained within the individual wells. Accompanying the target plate view is a reconstructed total ion chromatogram (see Figure 3).

The LC-MALDI software initially generated MS spectra for each sample spot. Following this screening for precursors, the unique peak picking software was employed, ensuring MS/MS data of candidates meeting the user defined criteria are acquired at the apex of the eluting chromatographic peak providing the highest quality spectra. These MS/MS spectra were subsequently submitted for Mascot® database searching in order to identify the protein in an automated fashion.

In a representative analysis, five individual proteins were identified from the mixture of proteins. The Mascot® search results are shown in Figure 7. Multiple peptides were identified for all of the proteins identified generating highly confident results. An example is shown in Figure 7 as an expansion of the Mascot® results from the 3rd ranked protein where *beta-lactoglobulin* is identified by five peptides.

An example of a typical MS spectrum from an iTRAQ™ LC-MALDI experiment is shown in Figure 4. The spectrum is derived from spot 123 of a 384 well MALDI target plate which equates to the location F8. The spectrum shows only a few peptide ions which illustrates an effective LC separation. The pooling of both samples with the isobaric isotopic labels gives the effect of doubling the intensity of the peptide signal detected in MS mode.

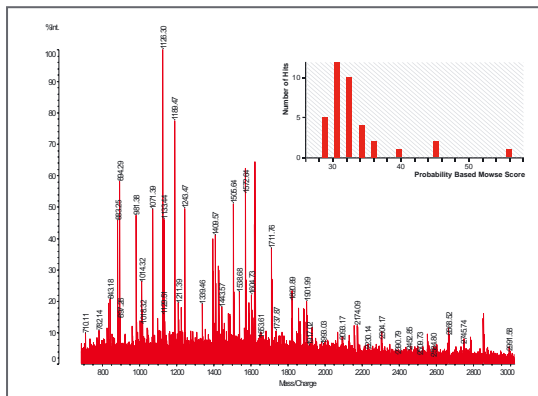


Figure 2. PMF spectrum of unseparated tryptic digest mixture of iTRAQ™ labelled 5 protein mixture and accompanying Mascot® search results showing no protein identification

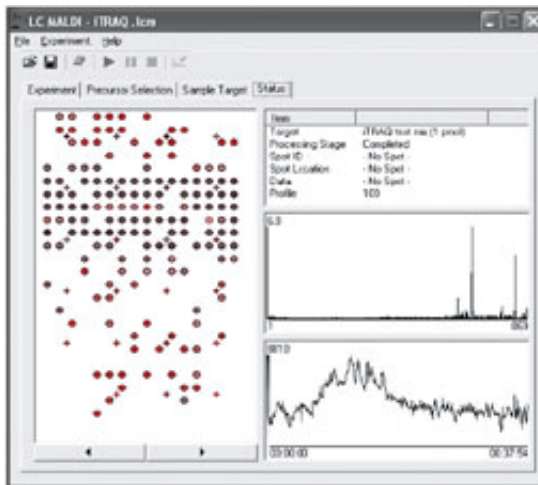


Figure 3. LC-MALDI software following the experiment showing MALDI target view, spectral view and reconstructed ion chromatogram

Figure 5 shows the CID enhanced MS/MS spectrum produced when the base peak in spot 123 was selected and fragmented (1204 m/z). Many fragment ion peaks are seen in the mass spectrum including those in the low mass region representing low mass sequence ions and immonium ions. The inset highlights the iTRAQ™ reporter ions at 114 and 117 Da which are used for relative quantification (ratios of their peak intensities or areas are compared). When submitted for database searching the MS/MS spectrum was identified as corresponding to a fragment of the protein *beta-lactoglobulin* with the amino acid sequence of **LDAINENK*** with a Mascot® score of 57.

